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GRANT NUMBER: DAMD17-94-J-4297

TITLE: Cloning of Human Monoclonal Antibodies Associated with Medullary Ductal Carcinoma

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REPORT DATE: September 13, 1995

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 13 1995	Annual 15 Aug 94 - 14 Aug 95

4. TITLE AND SUBTITLE
Cloning of Human Monoclonal Antibodies Associated with Medullary Ductal Carcinoma

5. FUNDING NUMBERS
DAMD17-94-J-4297

6. AUTHOR(S)
Dr. Peter Telleman

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
New England Deaconess Hospital
Boston, Massachusetts 02215

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

It is intended to identify and characterize newly expressed proteins (neo-antigens) which elicit plasma cell reactions in medullary and in non-medullary ductal carcinomas with circumscription and plasma cell infiltration. To accomplish this purpose, we exploit recently developed molecular procedures in a new application to derive antibodies from the tumor-infiltrating plasma cells, and then use these antibodies to retrieve the proteins whose new expression in the tumors induced the response. Ig sequencing of two patient MC samples revealed reiteration, supporting the presence of a focussed, specific immune response against an antigen by reactive plasma cells in the MC tumor. Additionally, phage Fab clones from these combinatorial phage libraries were shown to bind to HTB24 cells, the only available MC cell line, confirming cell surface expression of the putative neo-antigens on these cells. The neo-antigens will be identified by immunoprecipitation with reactive antibodies. Subsequently these proteins will be assesed for possible roles in the malignant prolifiration and as subjects for anti-breast cancer interventions.

14. SUBJECT TERMS
Medullary ductal carcinoma. Plasma cell. Breast cancer
Protein neo-antigens. Phage cloning.

15. NUMBER OF PAGES
16

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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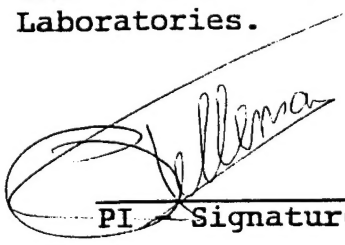

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INTRODUCTION.

Breast cancer presently kills more than 40,000 American women each year, second only to lung cancer as a cause of cancer death in women [1]. There is evidence for a small advantage in long term survival with chemotherapies in adjuvant settings [2], but there is little convincing evidence for systemic cures with any therapy where known residual metastatic disease exists. Thus, there is an enduring need for new approaches to treatment in breast cancer. In the search for such new approaches, it has been proposed by Lippman that "the most appropriate protein targets may emerge from a consideration of prognostic variables ... shown to be of value in clinical practice" [3]. The present work builds on this premise, to explore the molecular basis for the apparently improved survival in plasma cell-infiltrated medullary and other breast carcinomas, and specifically to describe the new breast carcinoma-associated proteins that elicit this response.

Medullary carcinomas (MC) are diagnosed in up to 5-7% of breast cancers [4]. MC is circumscribed grossly and microscopically without encapsulation, but its appearance is otherwise highly ominous, with large cells, abundant cytoplasm, large bizarre nuclei and frequent mitoses. Virtually all are histologic grade III, usually the worst prognostically, and they display a high degree of aneuploidy and typically lack hormone receptors. Yet patients with MC often do better than predicted for size and grade. Tumor is infiltrated and surrounded with lymphocytes and plasma cells; in its most exuberant expression, it was classically designated "medullary carcinoma with lymphoid stroma", prompting the off-stated impression that this tumor may be regulated by a host immune response.

It is our aim to derive antibodies from these plasma cells with specificity for malignant breast tissues using molecular techniques based on phage display libraries [5]. These powerful procedures can reconstruct immunoglobulin reactivities, even when sparsely expressed. Moreover, this approach maximally exploits the enrichment for reactivity and affinity for the tumor tissue which is inherent to the mature plasma cells, whose abundant Ig mRNA dominates over less mature and less affinity-selected B cells during the molecular cloning. The procedures are rapid and their products are truly human antibodies — without the attendant problems and limitations of human antibody technology (which will not immortalize plasma cells) [6].

STUDY PROGRESS

I) Library contruction.

In the original submission, we confirmed the IgG predominance of plasma cell infiltrations in one MCB specimen, demonstrated procedures for tumor disaggregation and cell separation, described a breast cell culture strategy we used, and obtained the HTB24 (MB157) medullary carcinoma cell line. We showed that we could succesfully amplify the genes from breast tumor plasma cells by PCR and that the pattern of λ amplifications suggested an oligoclonality to the Ig genes in the tumor, suggesting in turn a restricted immune response representation. In the period since the original proposal, we have obtained two additional medullary carcinoma samples for a total of three; we have prepared γ1,κ and γ1,λ libraries into M13 phage for two of these, with $>10^6$ members, well in excess of what we will need for effective representation in a restricted antibody library. We have devised procedure improvements to asses library quality and to streamline screening that will increase the power of the phage display method [7].

II) Unslected libraries - Random clone sequencing.

In a library of high diversity, there is no repetition of clones in any practical sized sample. In hyperimmunized individuals, antitetanus antibodies were present in a total B cell library in only 1:1000 to 1:5000 clones, and only 2/8 selected *positive* clones that were sequenced showed the same V gene usage [7]. In a further study with influenza-immunized mice, a dominant H chain and a dominant L chain were present in maximum proportions of 1:200 and 1:1000 in a total library, respectively [9]. Hence, any recurrence of genes in a limited random sample will signal that the library is highly

Table 1. Characteristics of H and L chain genes of random clones from an unselected library.

Group	L chain (κ)	Family	VK germline gene(putative)
I	p1, p15, p18, c10, c11, c17, c18, c19, c20	VK 1	NALM6
II	p9	VK 1	Vd
III	p7	VK 3	SU-DHL
IV	p12	VK 3	Vg
V	p4	VK 4	VKIV
	H chain	**Family**	**VH germline gene(putative)**
I	p1, c17, p18, c21	VH 1	DP-10
II	p7	VH 1	V1-03
III	p4, p12, p15	VH 3	1,9III
IV	c10	VH 3	V3-11
V	c18	VH 4	vhSP
VI	c22	VH 4	VH4.16

focussed and restricted.

In Ig sequencing of two patient MC samples, we were surprised by the already dramatic focus in the libraries, in the absence of *in vitro* panning. There was an apparent reiteration of sequences in these derived and diversified from single B cell clones (Table 1 summarizes VK and VH data from one donor; see also appendices A and B). The possibility of lab PCR or Fab-phage contaminants was ruled out by comparing against all other cloned Ig genes and phage-Fab in our laboratory. The possibility of a repeated plasmid clone was ruled out by the presence of different partners with each of the repeated chains, and by the different mutations present in the repeated clones in the different isolates. That the repeated clones of a given group are from a single original B cell is indicated by the minigene (CDR3) patterns in each group. We interpret the presence of one repeated clone among the Vκ sequences and two repeated clones among the VH as indicating that one of the original VL chains is missing; we expect that the other will be among the Vλ, to be examined shortly. Until single cell PCR studies are performed, we will not know the original VHVL pairings, but we may be able to surmise the general pairs by those showing best antigen reactivity, which should be apparent after sequencing the clones enriched on cell panning. Early data including 5 VH and 5 Vκ from a second patient's tumor clearly show reiteration as well (2/5 VH; 3/5 Vκ), but more sequences are needed to comment on commonality of gene usage between donors.

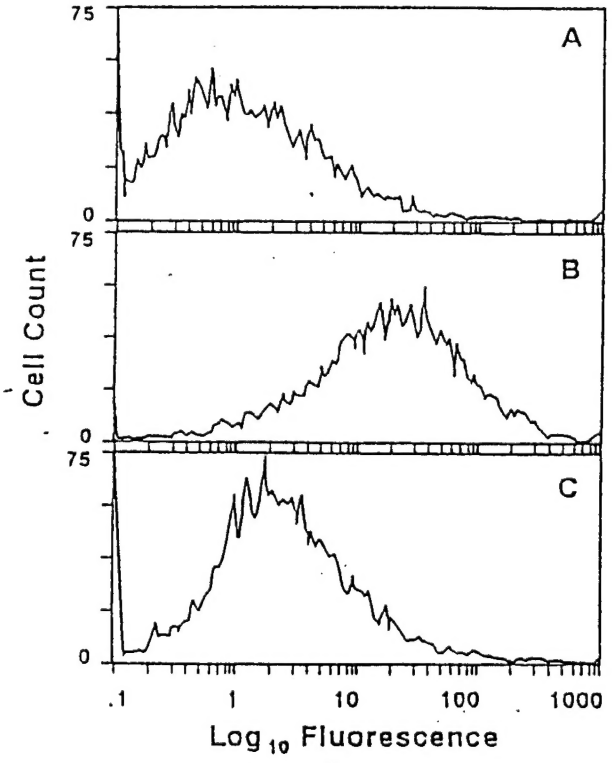

Fig.1. Flow cytometry of Fab-phage binding to cells. 2 x 10^{11} Fab-phage were inubated with 5 x 10^5 anti-Tac-expressing HD245 cells, washed, incubated with botinylated sheep anti-M13 antibody, then stained with avidin-phycoerythrin. A. Binding anti-tetanus toxoid Fab-phage (TT) to cells. B. Binding anti-idiotype Fab-phage (Id) to cells. C. As in B except preincubate Fab-phage with anti-Tac antibody, showing that binding is inhibitable and specific to cellular "antigen".

III) Library screening - Panning

Because our antigen is unknown, it is essential that our panning methods are optimized so that negative results are meaningful and so that enrichments of positive clones are effi-

cient, even with low antigen expression. Particle [8] and cell-based [9; A Griffiths, G Winter, unpubl.res.] selections have been applied in other systems, but these were with known antigens present in high concentrations. Furthermore, to our knowledge, prior cellular panning methods have used only erythrocytes, which are physically very different from plasma membrane cells of carcinomas which we wish to study.

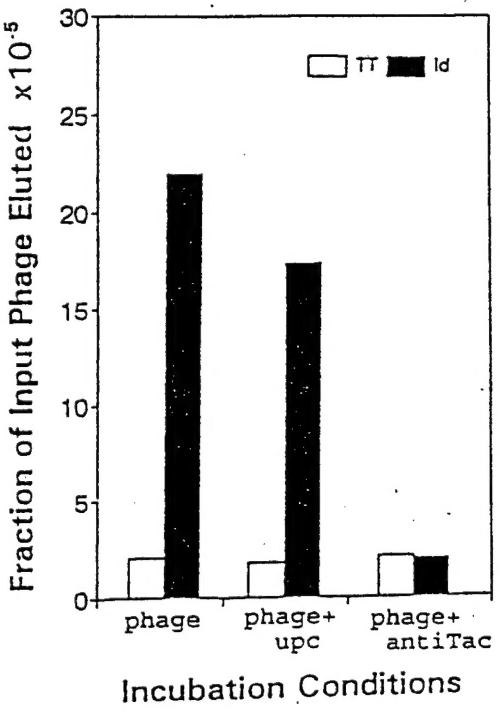

Fig.2. Enriched Fab-phage binding by specific cell-based panning. 10^{11} Fab-phage were incubated with 10^{6} HD245 cells for two hours, washed and eluted. Comparisons are between non-specific phage (TT) and specific phage (Id), and showing competition that is minimal with non-specific isotype matched antibody (+upc) but suppressed to background levels with specific antibody (+anti-Tac), demonstrating specificity.

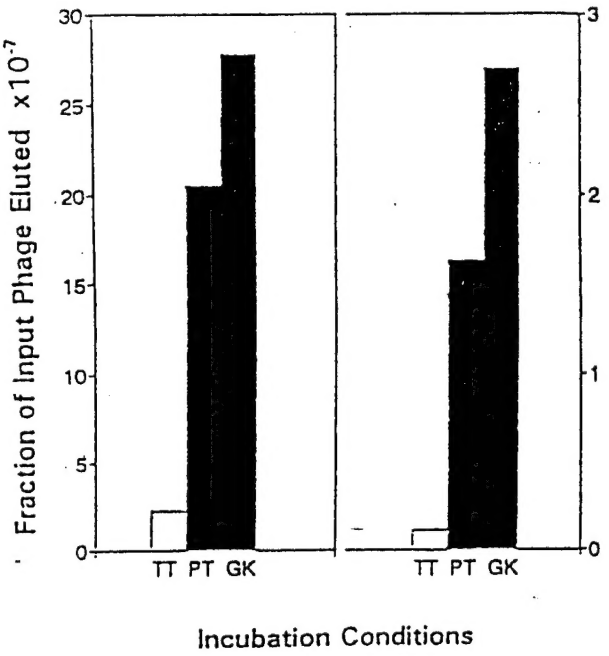

Fig.3. Enriched Fab-phage binding from patients libraries against HTB24 medullary carcinoma cells. Comparisons are between non-specific (TT) and patient libraries (PT and GK) of Fab-phage. Two different experiments are represented in which he details of the assay differed, but generally follow procedures of fig. 2. Each experiment showed significant better binding of Fab-phage from patient libraries than by non-specific phage.

We accordingly investigated optimal conditions for these pannings [J Watters, P Telleman & R Junghans, in prepn.], which required a model system with a cloned Fab-phage reactive with a cell surface molecule. We previously cloned an anti-idiotype Fab-phage from a combinatorial phage library derived from a patient treated with murine anti-Tac antibody [7]. This was tested against hybridoma cells expressing the anti-Tac antibody (HD245, gift of T Waldmann). This showed reactivity of Fab-phage by flow cytometry that was specific because excess unlabeled anti-Tac antibody suppressed phage binding to cells whereas a non-specific phage against tetanus toxoid (TT) did not react with HD245 (Fig.1).

With confirmation of surface expression of antibody on the hybridoma cell line, we proceeded to tests with panning and phage titer assessment. 10^{11} Fab-phage were mixed with 10^6 HD245 cells in 0.2 ml growth medium (GM) at 4°C for 2 hours, then spun at 1000 x g for 5 minutes. The pellet was resuspended in 1 ml cold GM and washed four times. Bound phage were diluted in HCl-glycine pH 2.2 and neutralized. This constitutes one cycle of panning. These phage are amplified in bacteria and the panning and elution are repeated. A ten-fold enrichement of anti-Id phage titer was observed relative to non-specific phage (TT) in one cycle of panning that was supressed to non-specific levels in the presence of competing "antigen" (anti-Tac antibody) (Fig.2. J. Watters, P. Telleman, and R. P. Junghans, in prep.). This is less than in pannings against purified proteins with standard methods [5], but sufficient for focussed, reduced-diversity libraries. These optimizations were recently completed. In the few days prior to this submission, we applied these techniques to perform panning of our two MC libraries ($\gamma 1,\kappa$ and $\gamma 1,\lambda$ combined) against HTB24 cells, the only available MC cell line. This showed 10- to 20-fold enrichments after a single cycle of binding to HTB24 relative to the TT Fab-phage in two different experiment on different days (Fig.3.). This observation is consistent with a library that is already highly enriched for antigen reactivity, which we estimate as 60% relative to the pooled VHVL libraries of one patient for whom extensive sequence data is available. This observation is an extremely important result and confirms surface expression of the putative neo-antigen, enabling all of the immunoprecipitation and gene cloning approaches as described in the original submission. To determine whether clones with certain H and L chain pairs are enriched by panning against HTB24 cells, we have recently developed an alternative way to distinguish clones: HaeIII fingerprinting. After panning, IgG H and L chain genes of random clones are amplified individually and restricted with HaeIII. The restriction fragments are seperated on a 0.8% polyacrylamide gel and grouped based on their restriction pattern. In contrast to sequencing , this method provides an easy and rapid way to test many clones for the presence of a particular H and L chain. Ultimately, enriched clones will be sequenced to determine whether they are derived from canonical initial B cell clones.

IV Protein antigen identification

A report by Colnaghi and co-workers [12] showed Her2/neu reactivity of EBV-transformed patient peripheral B cells when patients' tumors (i) overexpressed Her2/neu and (ii) were infiltrated. It is noted, however, that in 4/4 MC tumors tested, there was no Her2 overexpression [13; M. Press, pers. comm.], and it therefore seems unlikely that Her2/neu is in fact the principal eliciting antigen that gives MC its characteristic plasma cell infiltrates. Nevertheless, we pursue this for ease of performance and as a further approach to complement the primary methods. Even negative data are significant , to rule out this protein as the eliciting antigen in MC. We have recently obtained purified recombinant Her2/neu^{ECD} protein [14] for this purpose (gift of Dr. B. Fendly, Genentech). In a screening of several $\gamma 1,\kappa$ clones that appear to represent the repetoire focus, as well as the whole IgGκ and IgGλ libraries, were recently tested in a Her2/neu ELISA (Fig. 4a), and none was reactive, therefore establishing that the focussed part of the repetoir is not in response to Her2/neu.

A portion of breast carcinomas with mutated p53 may have serum antibodies to p53, but against non-mutated regions of the protein [15,16]. No data specifically tie this phenomenon to reactions in plasma cell infiltrated MC or NOS tumors. p53 is normally a nuclear protein, and does not fit our profile for a surface-expressed antigen that we postulated under Specific Aims in the original proposal, and it would not fit our MC cell line binding as described earlier. However, as stated for Her2/neu, reactivity to p53 is readily tested. We recently obtained purified GST-p53 fusion protein (gift of Dr. Dutta, HMS), to establish an ELISA for this test (Fig. 4b).

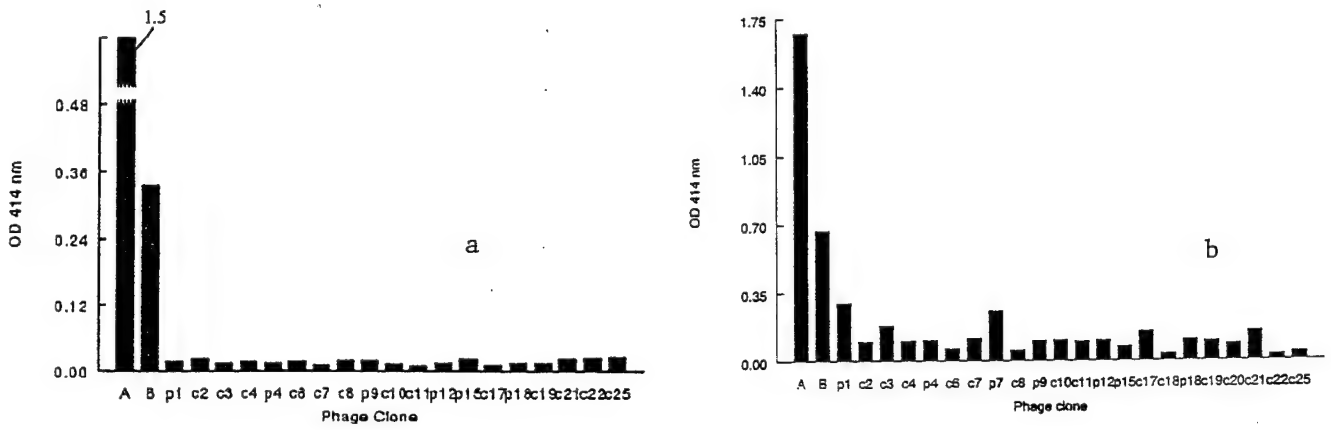

Fig. 4. Preliminary ELISA data of γ1,κ clones, representing the repetoire focus against a) Her2/neu and b) p53. With A: the positive control Her2/neu antigen with human anti-Her2/neu antibody or GST-p53 antigen with mouse anti p53 antibody, and B: tetanus-toxoid with Fab phage against tetanustoxoid.

V) Development of MC cell lines.

In a parallel effort to develop MCB cell lines, we tested two tumors for growth in scid mice, including one test with irradiated, estrogen-supplemented animals, but only one tumor showed initial growth that then regressed. It is known that malignant effusions are the best source of cells for generating tumor cell lines, and this was the source for the world's only MCB cell line. We have accordingly initiated a national search for pleural effusions fom patients with MCB diagnosis with announcements in the *Journal of Clinical Oncology, American Journal of Surgical Pathology, Oncology News International,* and other sources. We have also made contingency plans that would permit us to retrieve retrospectively the original antibody reactivities of those patients using old paraffin cell blocks and glass slides of such patients presenting with effusions long after their original presentation. As RNA will likely be too degraded in old samples from the plasma-cell infiltrated primary tumor tissue, we have designed a new strategy and new primers for PCR from the DNA of these tissues that will reconstruct the library based on the V(D)J recombinations at the immunoglobulin locus in the cells.

CONCLUSIONS.

Our sequence data support the premise that the MCB tissue will present a highly restricted antibody diversity as expected from a limited, specific response within the tissue. Furthermore, preliminary data indicate binding of Fab-phage clones from MC libraries to HTB24 cells, confirming cell surface expression of the putative neo-antigen(s) on these cells.

In the future these studies will enable (1) isolation of breast carcinoma-reactive Fab from plasma cell-infiltrated breast tissue, and (2) identification of breast carcinoma neo-antigens eliciting the plasma cell responses. Followup studies will build on these results to provide a detailed biochemical and molecular characterization of these neo-antigens, with further efforts to elucidate their role in tumorigenesis and potential for targeting through rationally designed strategies based on features of their actions.

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APPENDIX A

Kappa light chain sequences of random clones of a combinatorial MC library.

```
Framework I
NALM6:      GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACCATCACTTGC
p1k.cmp:    .G.GG.G..C.C.....................................T.....................  89%
c17k.cmp:   .G.GG.G..C.C.....................................T.....................  89%
c10k.cmp:   .G.GG.G..C.C.....................................T.....................  89%
c18k.cmp:   .G.GG.G..C.C.....................................C.....................  89%
c19k.cmp:   .G.GG.G..C.C.....................................................C.  89%
c20k.cmp:   .G.GG.G..C.C..T.....................................T.....................  88%
p15k.cmp:   .G.GG.G..C.C.....................................C.....................  89%
p18k.cmp:   .G.GG.G..C.C..............A..............G.....................  88%
p12k.cmp:   .G.GG.G..C.C..T.........G..A.........TTG...CC...G..A....C....C...T.C...  71%
c11k.cmp:   .G.GG.G..C.C..T.........CT..........TTGA..CCT.....A....C.T..C...T.C...  68%

CDR I
NALM6:      CGGGCAAGTCAGAGCATTAGCAGCTATTTAAAT
p1k.cmp:    ........................A...........                                    96%
c17k.cmp:   ........................A...........                                    96%
c10k.cmp:   ........................A...........                                    96%
c18k.cmp:   ........................A...C.......                                    93%
c19k.cmp:   ........................A...C.......                                    93%
c20k.cmp:   ........................A...........                                    96%
p15k.cmp:   ........................A...C.......                                    93%
p18k.cmp:   ........................C.......G...                                    93%
p12k.cmp:   A....C.........TG...........C...GCC                                     75%
c11k.cmp:   A...TC.........TG.....NNNNNNNNNNNN                                     48%

Framework II
NALM6:      TGGTATCAGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCTAT
p1k.cmp:    .............................................                          100%
c17k.cmp:   .............................................                          100%
c10k.cmp:   .............................................                          100%
c18k.cmp:   .............................................                          100%
c19k.cmp:   .............................................                          100%
c20k.cmp:   .............................................                          100%
p15k.cmp:   .....C.......................................                          97%
p18k.cmp:   ............................GC...............                          95%
p12k.cmp:   .....C..A.........T..CC.G..T..C.G......C.....                          77%
c11k.cmp:   NNNNNNNNNNNNNNNNNNNNN....T.....T.............                          48%

NALM6:      GCTGCATCCAGTTTGCAAAGT
p1k.cmp:    .....................                                                  100%
c17k.cmp:   .....................                                                  100%
c10k.cmp:   .....................                                                  100%
c18k.cmp:   .....................                                                  100%
c19k.cmp:   .....................                                                  100%
c20k.cmp:   ..............C..C...                                                  100%
p15k.cmp:   .....................                                                  100%
p18k.cmp:   ..........C..........                                                  95%
p12k.cmp:   .A.........ACAG.GCC.C.                                                 57%
c11k.cmp:   AG.........A...........                                                85%

Framework III
NALM6:      GGGGTCCCATCAAGGTTCAGTGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGCAGTCTGCAACCTGAAGATTTTGCAACTTACTACTGT
p1k.cmp:    ...................................................................................................  100%
c17k.cmp:   ...................................................................................................  100%
c10k.cmp:   ..........................................................G.............A..G..............T....  95%
c18k.cmp:   ..........................................................T.....................................CT....  96%
c19k.cmp:   ..........................................................T.....................................C..A..  96%
c20k.cmp:   ..........................................................C.....................................  98%
p15k.cmp:   ..........................................................T.....................................CT....  96%
p18k.cmp:   ......T.....................................................................................  98%
p12k.cmp:   ...A.....G.C.................G...........C.....................GTA.G.C.................GT...T.CA...  84%
c11k.cmp:   ..........G...................................................G.............A..G..............T....  94%

CDR III
NALM6:      CAACAGAGTTACAGTACCCCT
p1k.cmp:    .....................                                                  100%
c17k.cmp:   .....................                                                  100%
c10k.cmp:   ......G..C.....C....G                                                  80%
c18k.cmp:   ......G..C.....G.....                                                  85%
c19k.cmp:   ......G..C.....G.....                                                  85%
c20k.cmp:   .....................                                                  100%
p15k.cmp:   ......G..C..........                                                   90%
p18k.cmp:   .........G.........G                                                   90%
p12k.cmp:   ..G..CGTAGCA.C.CGG.TC                                                 33%
c11k.cmp:   ......G..C.....C....G                                                  80%

J Region

p1k.cmp:    TTCGG.CAA.GGACC.AG.TGG
c17k.cmp:   TTCGG.CAA.GGACC.AG.TGG
c10k.cmp:   TTTGG.CT..GGACG.AGCTGG
c18k.cmp:   TTCGG.CAC.GGACC.AG.TGC
c19k.cmp:   TTCGG.CAC.GGACC.AG.TGC
c20k.cmp:   TTCGGGG.AAGGG.CCAA..TG
p15k.cmp:   TTCGG.CAC.GGACC.AG.TGC
p18k.cmp:   TTTGG.CT..GGACC.AGCTGG
p12k.cmp:   TTCGG.G.A.GGACC.AG.TGG
c11k.cmp:   TTTTTGGCCTGGGG.C.AA.C.
```

APPENDIX B

Heavy chain sequences of random clones of a combinatorial MC library.

Group I

```
Framework I
1.9III:     CAGGTGCAGCTGGTGGAGTCTGGGGGAGGCGTGGTCCAGCCTGGGAGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTCAGT
p12h.cmp:   NNNCA..T...C.A.....................T.....................................................  90%
p15h.cmp:   NNNNNN.....C.C..................CT.....................................................A  87%
p4h.cmp:    NNNNNNNCAGCT.CTCGAGTC.............T.....................................................G  75%

CDR I
1.9III:     AGCTATGGCATGCAC
p12h.cmp:   .......CT......                                                                          86%
p15h.cmp:   .C.....CA......                                                                          80%
p4h.cmp:    .......CT......                                                                          86%

Framework II
1.9III:     TGGGTCCGCCAGGCTCCAGGCAAGGGGCTGGAGTGGGTGGCA
p12h.cmp:   ............CTC..............A............                                               90%
p15h.cmp:   ............CTCN...A.........A............                                               85%
p4h.cmp:    ............................A.....C......                                               95%

CDR II
1.9III:     GTTATATCATATGATGGAAGTAATAAATACTATGCAGACTCCGTGAAGGGC
p12h.cmp:   ........T.C...........C........TC.................                                       90%
p15h.cmp:   C.........C.............C...T...C...........C......                                       88%
p4h.cmp:    ........T.C...........C........A.C.................                                       90%

Framework III
1.9III:     CGATTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCTGAGGACACGGCTGTGTATTACTGTGCGAAA
p12h.cmp:   ...................................................................C.............................G.  97%
p15h.cmp:   ..............................................G..........T.........C.....................T....A...G.  93%
p4h.cmp:    ...................................................................C...........AA...C.C............G.  93%

CDR III

p12h.cmp      GATGGACGTAGTGGGACCTCCCGCCTGTACTACTTTGACTTC
p15h.cmp      GATGGACGTAGTGGGACCTCCCGCCTGTACTACTTTGACTTC
p4h.cmp       GACGGACCTAGTGGGACCTCCCAGTTATACTACTTTGACTCC
```

Group II

```
Framework I
DP-10:      CAGGTGCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCTGGGTCCTCGGTGAAGGTCTCCTGCAAGGCTTCTGGAGGCACCTTCAGC
p17h.cmp:   NNNNNNNNN..C.A......A.....................................................C.....................  85%
p18h.cmp:   NNNNNNNNNNNNCGAGC...A.....................................................................  80%
p1h.cmp:    NNNNNNNNNNNNNNNNNNNNNNNN.....................................................T.............  72%
c21h.cmp:   NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN.....................A.........................T  55%

CDR I
DP-10:      AGCTATGCTATCAGC
p17h.cmp:   ......A......A.                                                                          86%
p18h.cmp:   TA...........C.                                                                          80%
p1h.cmp:    ....G..........                                                                          93%
c21h.cmp:   TA...........C.                                                                          80%

Framework II
DP-10:      TGGGTGCGACAGGCCCCTGGACAAGGGCTTGAGTGGATGGGA
p17h.cmp:   ..........................T..............                                                97%
p18h.cmp:   ...........................................                                             100%
p1h.cmp:    ......................T....................                                             97%
c21h.cmp:   ...........................................                                             100%

CDR II
DP-10:      GGGATCATCCCTATCTTTGGTACAGCAAACTACGCACAGAAGTTCCAGGGC
p17h.cmp:   ...........................................                                             100%
p18h.cmp:   ......G.......G.........A......C.....................                                    92%
p1h.cmp:    ........................GGG.....................                                    94%
c21h.cmp:   ......G.......G.........A......C.....................                                    92%

Framework III
DP-10:      AGAGTCACGATTACCGCGGACGAATCCACGAGCACAGCCTACATGGAGCTGAGCAGCCTGAGATCTGAGGACACGGCCGTGTATTACTGTGCGAGA  100%
p17h.cmp:   ...................................................................T.....................G  97%
p18h.cmp:   .........A.....................................A.............C..........C.....................G  94%
p1h.cmp:    ...........................................................................T.....................G  97%
c21h.cmp:   .........A.....................................A.............C.....................................G  95%

CDR III

p17h.cmp      GGGGGGGACTGGTTCGACCC
p18h.cmp      GGGGGGGACTGGTTCGACCC
p1h.cmp       GGGGGGGACTGGTTCGACCC
p21h.cmp      GGGGGGGAGGGAACCC
```